IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Rabbani et al.	)
		)
Serial No.:	08/978,637	) Group Art Unit: 1635
		)
Filed:	November 25, 1997	) Examiner: J. Schultz
		)
For:	**COMPOSITION OF MATTER COMPRISING PRIMARY NUCLEIC ACID COMPONENT**	)

527 Madison Avenue (9thFloor)
New York, New York 10022-4304

DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

*Considered
1-20-05
JS*

Sirs:

1. I, Dakai Liu am an inventor of the subject matter claimed in the above-referenced application. My Curriculum Vitae is attached hereto as Exhibit 1.

2. I have reviewed the Office Action dated February 11, 2004 and particularly the enablement rejections on pages 5-8 .

3. I understand that the Examiner questions whether constructs containing antisense sequences once administered to a patient can be stably incorporated into said patient.

4. In the instant Declaration, I will present results showing that a construct containing an U1/anti HIV sequences is stably incorporated into three patients' cells for at least 48 months.

5. The retrovirus vector, HGTV43, used is shown in Exhibit 2. Specifically, HGTV43 contains LTR, packaging and ppt elements derived from Moloney murine leukemia virus, three U1 genes in their entirety. Each of these U1 genes does contain a C->T mutation at residue 4. Antisense sequences complementary respectively to the TAR region and against two separate sites of the TAT region of the HIV-1 genome are inserted within the RNA coding portion of the U1 gene.

6. Briefly, the following procedures were used. Each step will be described in detail below.

    a.   Preparation of producer cell lines containing a triple U1/anti-HIV construct;

    b.   Transduction of patients' PSBCs with retrovirus vector containing the triple U1/anti-HIV construct

    c.   Infusion of transduced cells

    d.   Assay PBMCs for the presence of antisense sequences

7. A producer cell line was obtained by transfecting a packaging cell line , NIH 3T3 cells capable of pseudotyping the human T cell leukemia virus II envelope protein with the sequence producing HGTV43 RNA (Figure A). The clones used are shown in Exhibit 3. Briefly, the packaging cell line was produced by transfecting with plasmids containing the Moloney murine leukemia virus (MMLV) gag-pol region (Miller et al., 1986, Mol. Cell. Biol. 6: 2895-2902) (Figure B), Gibbon ape leukemia virus (GaLV) env region (Trainor et al., 1984, Virology 137:201-205 (Figure C) and the coding sequence for the HTLV-II envelope (Figure D). Positive

transfectants were isolated using resistance to the antibiotic G418 according to the manufacturer's instructions (Gibco, Grand Island, N.Y.).

8. HIV positive patients between 18-50 years old having a CD4+ cell count of $\geq$200 cells/mm^3 and an HIV-1 viral load between 2,500 and 100,000 genome equivalents per ml participated in this study. The patients were administered granulocyte colony stimulating factor (GCSF) for four days. On day five, eight patients underwent leukapheresis and 5-10 x 10^{10} peripheral blood mononuclear cells (PBMCs) collected. The PBMCs expressing CD34+ antigen (PBSC) were positively selected using the Baxter Isolex 300 system following which the anti-CD-34-bead complex was eluted from the cells. The PBSCs were transduced with the HGTV-43 vectors containing high titers (10^4-10^6) antisense producing units /ml) supernatants in the packaging cell line described above. After one day, nonadherent cells were collected and adherent cells were trypsinized and the two cell fractions were pooled, washed twice with PBS and suspended (at a concentration of 1-2 x 10^7/ml). An aliquot was removed for measurement of HIV-1 using a standard p24 assay. The amount of HIV-1 p24 was compared with that before culture and transduction to make sure that the HIV-1 p24 concentration is $\leq$ titer determined at the beginning of the transduction period. The amount of antisense RNA in the cells was determined by RT-PCR.

9. Approximately 10 ml of 1-2 x 10^7 PBSC cells/ml were infused into each patient by venipuncture. The patients were monitored for adverse effects and viral loads, CD4+ T cell count and antisense RNA levels monthly for the first six months. No adverse effects were detected in any of the patients. Three patients dropped out of the studies six-nine months post-infusion. One patient (Patient F) dropped out of the study one year post infusion.

10. Samples were also taken between 9-48 months from the patients. Antisense RNA levels were measured in CD4+ and/or PBMC cells by RT-PCR. Specifically, the presence of antisense RNA in PBMC and CD4+ cells were determined by reverse transcription and PCR (RT-PCR) with specific U1/anti-HIV antisense primers. The real-time PCR amplification was monitored by a LightCycler™. The presence or absence of U1/anti-HIV-1 antisense RNA was determined by determining the size of the amplicon and electrophoresis gel-banding pattern after restriction enzyme digestion of the amplicon and the electrophoresis gel-banding pattern after restriction enzyme digestion of amplicon. Additionally, the presence or absence of the U1/anti-HIV-1 antisense RNA was determined in bone marrow CD34+ cells nine months after infusion as a measure of the success of autologous transplantation of CD34+ stem cells into the patient.

11. Preliminary results for five patients tested are described in Liu et al., 2002, Mol. Ther. 5(5):S254, Liu et al., 2001, Mol. Ther. 3(5):S309, Liu et al., 2000, Mol. Ther. 1(5):S147 (Exhibit 4). It appears that all of the subjects tested expressed anti-HIV-1 antisense RNA in CD4+ cells nine months after infusion.
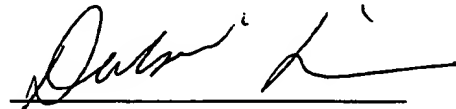
12. Further results for the five patients are shown in Table I, attached hereto.

13. In conclusion HIV-1 antisense RNA was detected in all five patients tested for at least 6-9 months in CD4+, PBMCs and CD34+ cells. HIV-1 antisense RNA was detected in cells from three of the patients for 48 months. Thus, these results indicate that constructs containing antisense sequences once administered to a patient can be stably incorporated into said patient.

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 10/20/04

Dakai Liu

NAME	POSITION TITLE
Dakai Liu	Director of Enzo Therapeutics Gene Therapy

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR	FIELD OF STUDY
Hubei Medical University, Hubei, P.R. China	M.D.	1983	Medicine & Medical Virology
Albert Einstein College of Medicine, Bronx, NY, U.S.A.	Ph.D.	1994	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE

1978-1982	Medical course work, Hubei Medical University, Hubei, P.R. China.
1982-1983	Medical Internship at the Wuhan Hospital No. 5.
1983-1984	Medical Residency, Dept. of Pediatrics, No. 1 Hospital affiliated with the Hubei Medical University, Hubei, P.R.C.
1984-1986	Research Fellowship, Virology Department of the Pediatric Institute, Hubei Medical University, Hubei, P.R.C. - Immunologic pathology of the respiratory syncytial virus (RSV).
1986-1988	Assistant Professor, Dept. of Virology and Molecular Biology, Wuhan Univ., Wuhan, P.R. China - Research on poliovirus epidemiology.
1989-1994	Ph.D. thesis research, Dept. of Cell Biology, Albert Einstein College of Medicine - Regulation of mouse H1 histone gene expression during differentiation.
1994-1996	Assistant Investigator, Enzo Therapeutics, Inc., New York. Design and development of intracellular vehicle for expression and delivery of genetic antisense RNA.
1996-1997	Associate Investigator, Head of Cell Biology Lab, Enzo Therapeutics, Inc. Design and development of cell-specific targeting retrovirus vector and its producer cell line.
1997-1998	Senior Scientist, Enzo Therapeutics, Inc. Design and development of additional cell-specific targeting retrovirus vectors and producer cell lines.
1998-present	Director of Gene Therapy, Enzo Therapeutics, Inc.
	Co-Investigator, clinical trial, "Evaluation of the Safety and Effects of *Ex Vivo* Modification and Re-infusion of CD34$^+$ Cells by an Antisense Construct Against HIV-1 in a Retrovirus Vector."

HONORS AND AWARDS

1979, 1980, 1981	Distinguished Student Award, Hubei Medical University.
1987	Outstanding Instructor Award, Wuhan University.
1988-1994	Sue Golding Graduate Scholarship, Albert Einstein College of Medicine.

GRANT AWARDS

1998	Principal Investigator, NIAID SBIR Research Grant No. 1 R43 AI40827-01A1. *Ex Vivo* Antisense Therapy for HIV Infection.

PUBLICATIONS

Liu, D., Conant, M., Cowan, M., Laurence, J., Eden, C., Dunn, E., Thalenfeld, B., Engelhardt, D.L., Engraftment and Development of HGTV-43-Transduced CD34+ PBSC in HIV-1 Seropositive Individuals. *Mol. Ther.* 5(5): S254, 2002

Liu, D., Wu, Q., Dunn. E, Eden, C., Cowan, M., Conant, M, Laurence, J., Thalenfeld, B., Engelhardt, D.L, Development and maturation of U1/anti-HIV-1 antisense RNA-expressing CD4+ cells from transduced CD34+ PBSC. *Mol. Ther.* 3(5):S309, 2001

Liu, D., Wu, Q., Ng, J., Criscuolo, E., Felix, E., Chu, S., Wren, E., Cowan, M., Eadon, C., Conant, M., Thalenfeld, B., Engelhardt, D.L., Successful Engraftment of Transduced CD34+ Cells and Continued Expression of Genes Encoding Anti-HIV-1 Antisense RNA in HIV-1-Infected Human Subjects. Abstract of the 8th Conference on Retroviruses and Opportunistic Infections, Chicago, IL: February 4-8, 2001.

Liu, D., Wu, Q., Ng, J., Ho, J., Dunn, E., Cowan, M., Conant, M., Thalenfeld, E., Engelhardt, D.L., *In Vivo* Maturation of U1/Anti-HIV-1 Antisense RNA-expressing CD4+ Cells from Transduced CD34+ PBSC in HIV-1 Seropositive Individuals. *Mol. Ther.* 1(5):S147, 2000

Liu, D., Ng, J., Ho, J., Wu, Q., Nuovo, G., Wren, E. Cowan, M.J. Conant, M.A., Thalenfeld, B.E., Engelhardt, D.L. A novel retrovirus-based transducing vector that transduced CD34+-enriched leukocytes. Abstracts of the 7th Conference on Retroviruses and Opportunistic Infections, San Francisco, California: January 30-February 2, 2000.

Engelhardt, D.L., Liu, D., Richardson, J., Laurence, J., Ng, J., Cowan, M.J. Broad spectrum resistance to HIV-1 strains by antisense sequences expressed in CD4+ cells. Abstracts of the XII World AIDS Conference, Geneva:34, June 28, 1998.

Engelhardt, D.L., Gulati, S.C., **Liu, D.**, *et al*. Use of gene therapy in inhibiting HIV growth. Abstracts of Acta Haematologica 10th Symposium on Molecular Biology of Hematopoiesis, Hamburg, Germany, July 2-6, 1997.

Liu, D., Donegan, J., Nuovo, G., Mitra, D., Laurence, J. Stable human immunodeficiency virus type 1 (HIV-1) resistance in transformed CD4+ monocytic cells treated with multitargeting HIV-1 antisense sequences incorporated into U1 snRNA. *J. Virol.* 71:4079-4085, 1997.

Liu, D., Donegan, J., Kelker, N., Nuovo, G., Laurence, J. High level and stable HIV-1 resistance in CD4+ cells by multi-targeting antisense RNA incorporated into U1 snRNA. Abstracts of the XI International Conference on AIDS:27, Vancouver, 1996.

Dong, Y., **Liu, D.**, and Skoultchi, A.I. An upstream control region required for inducible transcription of the mouse H1° histone gene during terminal differentiation. *Molec. Cell. Biol.* 15:1889-1900, 1995.

Liu, D. Cloning, characterization and regulation of mouse H1 histone genes. (Ph.D. Dissertation), Albert Einstein College of Medicine, Yeshiva University, 1994.

Liu, D. Research progress in human immunodeficiency virus. *Trends in Biol. Sci.*, China, 27:16-19, 1989.

Liu, D., Zhang, C., Liu, M., Wan, R., and Guo, S. Development and application of ELISA for RSV detection. *J. Microbiol.*, China, 8:46-48, 1988.

Liu, D., Zhang, C., Liu, M., Wan, R., and Guo, S. The level of serum histamine and its significance in children with RSV infection. *J. Immunol.*, China, 4:160-164, 1988.

Liu, D. and Zhu, L. The value of serum C-reactive protein in distinguishing viral and bacterial infection. *J. Immunol.*, China, 4:221, 1988.

Liu, D. *Medical Virology* Wuhan University Press, China, 1988.

Liu, D. and Ding, J. *Experiments in Virology* Wuhan University Press, China, 1987.

Liu, D. Study of children's immune response to respiratory syncytial virus (RSV) and development of enzyme-linked immunosorbent assay for RSV antigen in nasopharyngeal secretion. (M.D. Dissertation), Hubei Medical University, China, 1985.

PATENTS

Liu, D., Rabbani, E., Lyubarsky, L., and Ng, J. Vectors and viral vectors, and packaging cell lines for propagating same. U.S. Patent Application, Serial No. 08/822,963, filed March 21, 1997.

Rabbani, E., Stavrianopoulos, J.G., Donegan, J.J. **Liu, D.**, Kelker, N., and Engelhardt, D.L. Novel property effecting and/or property exhibiting compositions for therapeutic and diagnostic uses. U.S. Patent Application, Serial No. 08/574,443, filed December 15, 1995.
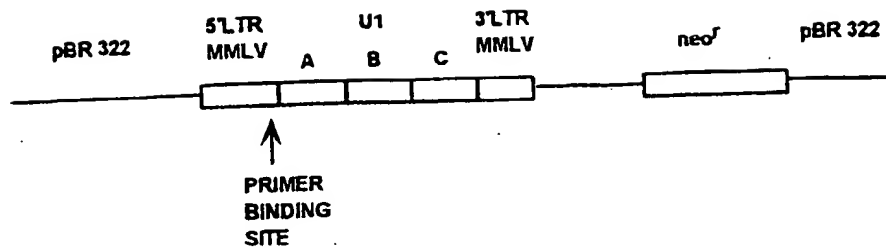
CLINICAL TRIAL

Principle Investigator: Morton J. Cowan, M.D., Co-Principle Investigator: Marcus A. Conant, M.D., Co-Investigators: **Dakai Liu, M.D., Ph.D.**, Evaluation of the Safety and Effects of *Ex Vivo* Modification and Re-infusion of CD34$^+$ Cells by an Antisense Construct Against HIV-1 in a Retrovirus Vector. FDA Investigational New Drug No. 7457, 1997.

Principle Investigator: Morton J. Cowan, M.D., Co-Principle Investigator: Marcus A. Conant, M.D., Co-Investigators: **Dakai Liu, M.D., Ph.D.**, Evaluation of the Safety and Effects of *Ex Vivo* Modification and Re-infusion of CD34$^+$ Cells by an Antisense Construct Against HIV-1 in a Retrovirus Vector. Recombinant DNA Activities Committee (RAC) Human Gene Transfer Protocol #9801-230, 1998.
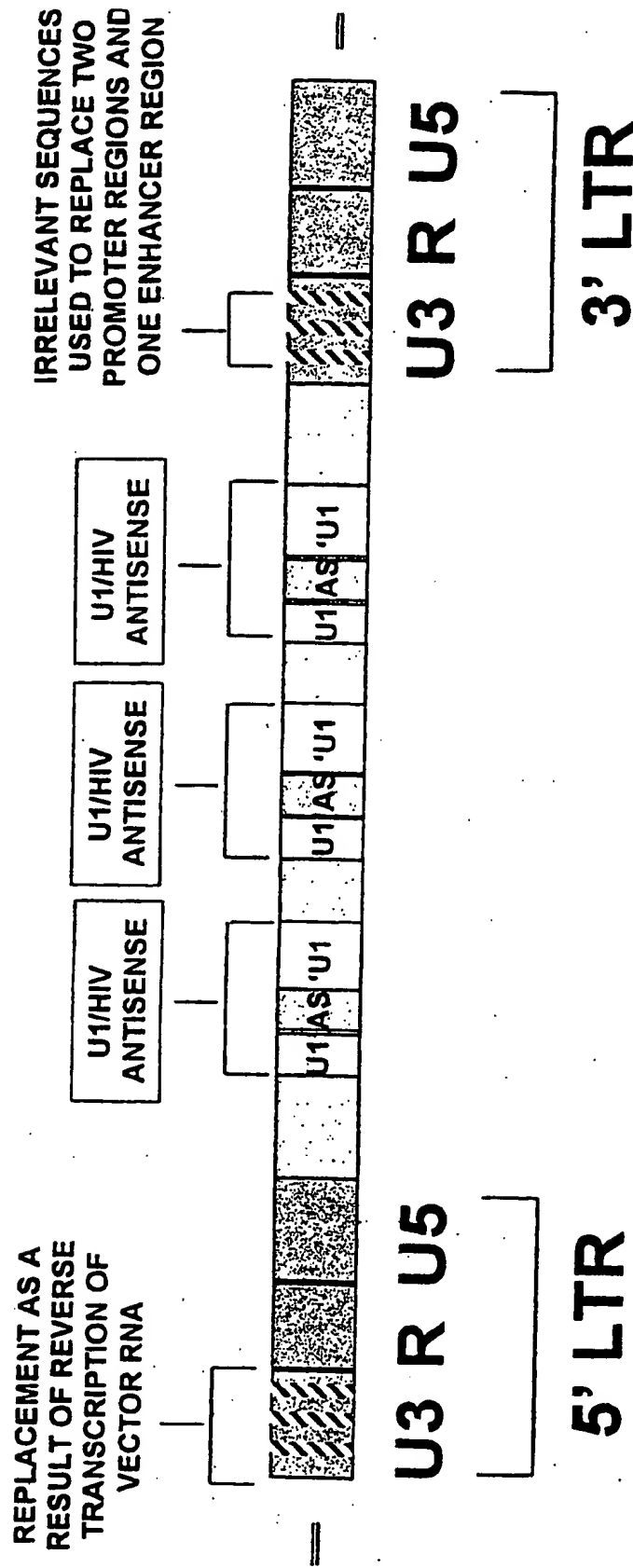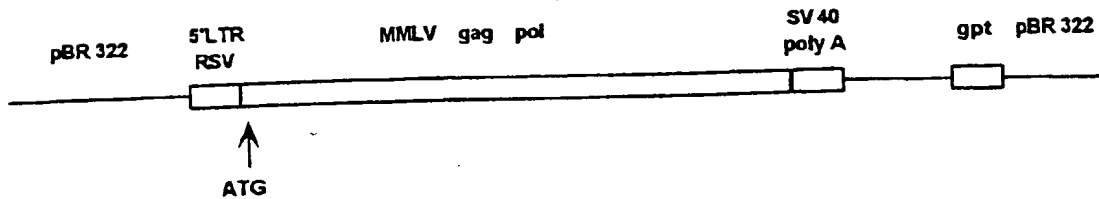
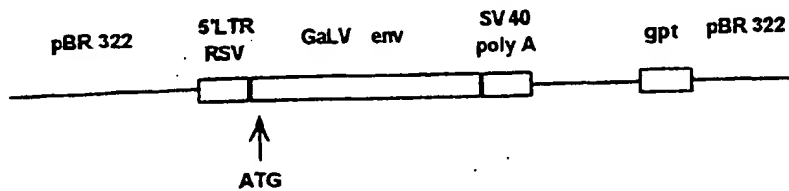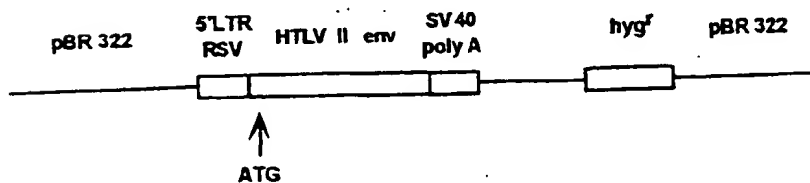FIGURE 7: Retrovirus Vector with Inactivated Promoter/Enhancer and Containing Three U1/HIV Antisense Cassettes

A

5'LTR MMLV U1 3'LTR MMLV

pBR 322   A B C   neo^r   pBR 322

PRIMER
BINDING
SITE

B

pBR 322   5'LTR RSV   MMLV gag pol   SV 40 poly A   gpt pBR 322

ATG

C

pBR 322   5'LTR RSV   GaLV env   SV 40 poly A   gpt pBR 322

ATG

D

pBR 322   5'LTR RSV   HTLV II env   SV 40 poly A   hyg^r   pBR 322

ATG

A. Triple U1 Construct;
B. gag-pol Construct;
C. GaLV Envelope Construct; and
D. HTLV II Envelope Construct.

enhancing cytolysis mediated by natural killer (NK) cells and cytotoxic T lymphocytes (CTL). It is therefore a reasonable cytokine to use in order to augment vaccine-induced immune responses. We investigated the ability of recombinant SV40 (rSV40) vectors encoding murine IL-12 (SV(mIL-12)) to enhance immune responses against gp120 in mice co-immunized with rSV40 encoding HIV-1$_{NL4-3}$ gp120 (SV(gp120)). Methods SV(gp120) and SV(mIL-12) were produced by cloning cDNAs for HIV-1$_{NL4-3}$ gp120 and murine IL-12 respectively into Tag-deleted SV40 genomes. In SV(mIL-12), the p35 and p40 subunits of IL-12 were separated by a poliovirus IRES. Cloned, stably-transfected, P815 cells expressing cell membrane gp120 were used as targets in a cell-based ELISA (CELISA) to assay antibody responses against gp120. The same cells were also used as targets to measure gp120-specific cytotoxic lymphocyte responses in ^{51}Cr-release assays. Gp120 expression on target cells was verified by flow cytometry. Mice were injected at monthly intervals with SV(mIL-12) and/or SV(gp120). In combination regimens, the IL-12 vector was given 3 days before the gp120 vector, or vice-versa. Control mice received SV(HBS), which encodes hepatitis B surface antigen. Results Following a single inoculation of gp120 +/- IL-12, none of the mice made detectable antibody responses assayed by CELISA. Co-administration of IL-12 however, affected anti-gp120 responses by unselected popliteal lymph node and splenic cytotoxic lymphocytes. Mice that received 2 treatments with SV(gp120) alone, or staggered inoculations with SV(mIL-12) given before or after SV(gp120), made very powerful anti-gp120 cytolytic responses (≥60% specific lysis, measured at effector:target ratios of 10:1 and 20:1). IL-12 alone did not elicit specific cytolytic activity. Conclusions Although a single immunization with gp120 +/- IL-12 did not elicit detectable humoral immunity against gp120, IL-12 strongly augmented the cell-mediated responses in immunized animals. Furthermore, the immunization regimen itself appears to dramatically alter the ability of immunized mice to mount a specific cytolytic response against gp120: specific anti-gp120 cytotoxic lymphocyte responses were very high in mice given 2 immunizations with SV(gp120) alone, and even higher in staggered combinations with SV(mIL-12). These results suggest that rSV40s may be useful vehicles for delivering HIV vaccines, and that cell-mediated immune responses may be enhanced by including immunostimulatory cytokines such as IL-12.

777. Internalization and Recycling of APJ, an HIV-1 Co-Receptor, in Transfected Cells and Human Neurons

R. J. Pomerantz,[1] X. Fan,[2] M. Mukhtar,[1] G. C. DuBois,[2] N. Zhou.[1]
[1]Dorrance H. Hamilton Laboratories, Center for Human Virology, Division of Infectious Diseases, Department of Medicine, Jefferson Medical College; [2]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA.

The human receptor APJ, a member of the G protein-coupled seven-transmembrane receptor family, has been shown to serve as a co-receptor for the entry of human immunodeficiency virus (HIV)-1 and simian immunodeficiency virus and it dramatically expressed in central nervous system (CNS) based cells. In this study, expression of APJ tagged with the green fluorescent protein (GFP) and a fluorescent peptide (FITC conjugated apelin13) were used for studying receptor internalization, and recycling, in real-time, in stably expressing cells and neuron cells. Fusion of the C-terminus of APJ to the N-terminus of the GFP did not alter receptor ligand binding and functions including signaling and internalization. Using 293 cells stably expressing APJ-GFP, we found that a rapid internalization of APJ receptor was induced by stimulation of both ligands, apelin36 and apelin13, in dose-dependent manner, but only apelin36 could inhibit the cell-cell fusion involved with APJ and HIV-1 gp120 (89.6). The intact cytoplasmic C-terminal domain was found to be required for ligand and phorbol ester (PMA)-induced APJ internalization. Furthermore, investigations showed that the internalized APJ was co-localized with transferring receptors in clusters of vesicles and was recycled to cell surface after removal of ligand. The result with cell-cell fusion involved with APJ and 89.6 gp120 indicated that the gp120 of 89.6 was able to bind to APJ, but the gp120 of 89.6 could not induce the internalization of APJ receptor. The CNS represents a unique reservoir site for HIV-1. As such, molecular therapeutics to inhibit HIV-1 entry and fusion via this unique CNS receptor is now able to be rationally-designed.

778. Engraftment and Development of HGTV43-Transduced CD34$^+$ PBSC in HIV-1 Seropositive Individuals

Dakai Liu,[1] Marcus A. Conant,[2] Morton J. Cowan,[2] Jeffrey Laurence,[3] Christopher Eden,[2] Elizabeth Dunn,[2] Barbara E. Thalenfeld,[1] Dean L. Engelhardt.[1]
[1]Enzo Therapeutics, Inc., Farmingdale, NY, United States; [2]University of California, San Francisco, San Francisco, CA, United States; [3]New York Presbyterian-Cornell Medical Center, New York, NY, United States.

HIV-infected subjects who have undergone one-time ex vivo.CD34$^+$ stem cell (PBSC) transduction and transplantation continue to produce cell populations expressing anti-HIV-1 antisense RNA up to 24 months post infusion. Successful engraftment is demonstrated by detection of anti-HIV-1 RNA in bone marrow CD34$^+$ cells. The presence of the transgene in circulating PBMC is confirmed by detection of anti-HIV-1 antisense RNA in these cells, as well as CD4$^+$ subpopulations. Using this gene RNA therapy approach, a long-term source of anti-HIV-1 antisense RNA producing lymphocytes has been established.

A retrovirus vector HGTV43 was used to deliver a set of genes encoding U1/anti-HIV-1 antisense RNA targeting *tar* and two separate sites of *tat/rev* region in the HIV-1 genome. These antisense RNAs have been shown to provide protection against HIV-1 infection *in vitro* in the monocytic lymphoma cell line U937. Approximately 10^8 CD34$^+$ cells from 5 individual subjects were transduced and infused autologously with the HGTV43 vector.

Engraftment and development of transduced CD34$^+$ PBSCs *in vivo* have been monitored by real-time RT-PCR detection of anti-HIV-1 antisense RNA expression in PBMCs, peripheral blood CD4$^+$ cells (>95% purity), and BM-derived CD34$^-$ cells (including CD34$^+$/DR$^-$ cells). The identity of the U1/anti-HIV-1 antisense RNA-specific RT-PCR amplicon has been confirmed by restriction enzyme digestion followed by gel electrophoresis.

In summary, no subject has shown any serious adverse event that could be attributed to the procedure. HGTV43-transduced CD34$^+$ PBSC were engrafted and developed in vivo into mature CD4$^+$ lymphocytes in HIV-1 seropositive individuals, and these cells continued to express anti-HIV-1 antisense RNA for 24 months to date, which is the longest example of gene expression in hematopoietic cells in non-ablated adults. This procedure will be expanded to include strategies to enhance engraftment and survival of the engineered stem cells. These strategies will be tested for safety and efficacy in a new set of clinical trials.

779. Improved Design of Oncoretroviral Vectors for the Delivery of HIV-1 TAT and Rev RNA Decoys

Stephen E. Braun,[1] Fay E. Wong,[1] Lorrin Lee,[1] Julianna Lisziewicz,[2] R. Paul Johnson.[1]
[1]Div. of Immunology, NERPRC, Harvard Medical School, Southborough, MA, United States; [2]Research Institute for Genetic and Human Therapy, Washington, DC, United States.

RNA decoys have a number of advantages for the inhibition of HIV replication, including their lack of immunogenicity and their ability to target conserved genes essential for viral replication. However, optimal inhibition of viral replication by RNA decoys has generally been obtained with multimeric RNA decoys, often consisting of 25 to 50 copies, which significantly increase the risk of transgene instability when delivered using retroviral vectors. We therefore examined a number of parameters affecting the ability of oncoretroviral vectors to stably deliver HIV-1 RNA decoys and inhibit viral replication. To facilitate subsequent evaluation of an optimized construct in a primate model, we examined the ability of these constructs to inhibit simian immunodeficiency virus (SIV) replication. Because of the conservation of Tat/TAR interactions among primate lentiviruses, HIV-1 TAR decoys can effectively inhibit transactivation by the SIV Tat protein. For the retroviral backbone, we chose the oncoretroviral vector MMP, which does not contain a selectable marker gene, and generated a series of vectors with and without intact splice donor (SD) and splice acceptor (SA) signals, and with the oncoretroviral LTR or an internal HIV-1 LTR transcriptionally regulating the polymeric TAR and RRE RNA decoys. As an initial assessment of inhibitory activity, these vectors were transiently transfected into 293T

strongly support the hypothesis that liver cells other than hepatocytes themselves can give rise to liver regeneration.

Our present study is to characterize the stem cell surface markers for the DDC oval cells and the growth factors that are induced in the DDC diet treated liver.

ADVANCES IN CLINICAL GENE THERAPY

870. In vivo maturation of U1/anti-HIV-1 antisense RNA-expressing CD4+ cells from transduced CD34+ PBSC in HIV-1 seropositive individuals

Dakai Liu*, Qi Wu*, Elizabeth Dunn†, Christopher Eden†, Morton J. Cowan†, Marcus A. Conant†, Jeffrey C. Laurence‡, Barbara E. Thalenfeld*, Dean L. Engelhardt*
*Enzo Therapeutics
†University of California San Francisco
‡New York Presbyterian-Cornell Medical Center

A transducing vector, HGTV43, was developed to deliver three U1/anti-HIV-1 antisense RNA genes into CD34+ cells. This vector contains LTR, packaging and ppt elements derived from Molony murine leukemia virus, is pseudotyped with the envelope protein from HTLV2 virus, and contains no known promoter other than that from the U1 RNA-producing gene. The targets for this anti-HIV-1 antisense RNA include a portion of the TAR gene and two separate sites of the TAT region of the virus. When this vector was used to transduce human CD34+ cells, anti-HIV-1 antisense RNA was detected in the cells within 18 hours after initiation of transduction. The antisense RNA produced in the transduced cells was equal to 10% of that produced in a highly HIV-1 resistant cell line containing these three anti-HIV-1 antisense genes.

Five HIV-1 infected subjects are currently enrolled in a clinical trial using this vector. Approximately 1010 peripheral blood mononuclear cells were collected by leukapheresis from these subjects after granulocyte stimulating factor treatment. After CD34+ cell enrichment on an Isolex 300i column, ~108 CD34+ enriched cells were transduced with our HGTV43 vector. These transduced cells were then infused autologously. .

The end-points of the trial included the safety of this protocol, the determination of the presence or absence of antisense RNA in the peripheral blood mononuclear cells and in the CD34+ cells in bone marrow samples taken between 6 and 9 months after infusion, viral load and CD4+ count. In addition, for all of these end points, in the course of the trial we began to assay for the presence of anti HIV-1 antisense RNA in the circulating CD4+ cell population. The anti HIV-1 antisense RNA was assayed using an RT/PCR format, where the PCR was performed in a Roche Light Cycler. The amplicon product was further characterized using restriction enzyme analysis.

No subject had a serious adverse event that could be attributed to the procedure. All 5 subjects were shown to express anti-HIV-1 antisense RNA at some time during the 5th or 6th month after infusion, and one subject expressed anti-HIV-1 antisense RNA in the peripheral blood mononuclear cells and CD4+ cells more than a year after infusion. Anti-HIV-1 antisense RNA continues to be produced in all subjects. Four out of five subjects had anti-HIV-1 antisense RNA in the bone marrow CD34+ cells. (The fifth subject did not yield enough CD34+ cells to make the assay valid and was thus recorded as QNS or quantity of cells not sufficient to perform the assay.) All 5 of the subjects had anti-HIV-1 antisense RNA in the CD4+ cell population at the end of the trial. Monitoring of these patients is continuing in follow up.

These results are a significant and perhaps unique example of the survival of transduced stem cells in nonablated adult human subjects with continued transgene activity as well as with replication and differentiation of these engineered cells into the CD4+ pool.

871. A phase 1 clinical trial of intra-arterial adenovirus p53 (SCH 58500) gene therapy for colorectal tumors metastatic to the liver

Isabella Atencio*, R. Warren†, A.P. Venook†, M.M. Kemeny‡, C. Staley§, D. Fraker¶, J. Horowitz‖, M. Rybak‖, S. Freeman‖, S. Indelicato‖, M. Grace‖, L. Xie‖, K. Kolz‖, M. Musco‖, R. Bordens‖, M. Fritz‖, S. Swanson‖, S. Jacobs‖, C. Cullen‖, E. Burke‖, J. Bitsura‖, S.F. Wen*, J. Shinoda*, B. Hutchins*, D. Maneval*
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§Emory University
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Purpose: The p53 gene is frequently defective or deleted in colorectal cancer, where restoration of wild-type p53 function can suppress tumor growth. We report the use of a replication-deficient recombinant human adenovirus encoding p53 (SCH 58500) delivered via the hepatic artery to evaluate the potential for p53 gene therapy in a Phase 1 study of colorectal cancer patients with metastatic disease in the liver. Key objectives of this study included evaluation of patient safety, adenoviral shedding, and evidence of gene transfer and expression. Materials and Methods: A single dose of SCH 58500 was administered angiographically to the right or left hepatic lobe. Patients (N=30) were scheduled for laparotomy within 2-7 days for either hepatic arterial pump replacement or liver resection. Two to six patients were treated at one of 9 dose levels, beginning at 7.5E+09 particles/dose with escalation to 7.5E+13 particles/dose. Serum samples were collected for analysis of antibodies to SCH 58500, and biopsies of tumor and normal liver from both treated and untreated lobes were analyzed for gene delivery and transgene expression. Additional cohorts of 9 patients received multiple injections of SCH 58500, in 1-3 monthly cycles, dosing at 7.5E+11 to 2.5E+13 particles/injection. 7 patients received multiple injections of SCH 58500 with chemotherapy (FUDR). Results: A severe adverse event associated with hemodynamic changes was observed mid-infusion in a patient scheduled to receive a single dose SCH 58500 at 7.5e13 particles. This patient recovered. This dose was defined as the dose limiting toxicity (DLT). All other patients were treated without DLT. Blood chemistry showed elevated liver functioning tests (LFT), fibrinogen split product (FSP) and PTT with decreased platelets at doses greater than 7.5E+11 particles, which were in most cases clinically insignificant, as none of the patients experienced clinical adverse events from these changes. Total lymphocyte counts fell transiently in all patients within 12 hours of administration, and rebounded within 7 days post-treatment. Transient transaminase elevations were seen in a majority of patients post-treatment. Potentially related adverse events included low grade fevers, decrease in blood pressure, headaches, myalgia, chills, and nausea. Elevations of anti-adenovirus antibody titers were detected within 1-3 weeks of dosing. Transgene expression, detected by RT-PCR, increased with dose and was detected more frequently in liver than in tumor specimens. Laser scanning cytometry detected apoptosis in tumor, but not normal tissues. No infectious viral shedding was detected. No radiographic responses were seen. Conclusion: We conclude that the maximum tolerated dose of SCH 58500 after administration into the hepatic artery is 2.5E+13 particles, and that regional delivery results in detectable transgene expression in normal and malignant tissues in the liver.

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delivered in this fashion include single chain antibodies, dominant negative mutants of important HIV-1 proteins, ribozymes, decoys, intrakines, and others. Limitations of retroviral gene delivery include relatively low vector concentrations and transduction efficiencies, the need to simulate target cells ex vivo, etc. We devised recombinant viral vectors based on large T antigen-deleted SV40 genomes (rSV40) and applied these vectors to the experimental therapy of immunosuppressive lentivirus infection in vitro. The strengths of rSV40 vectors for this purpose include high production titers (up to 10(12) infectious units (TU)/ml), high transduction efficiency (>95%) without selection, ability to transduce both dividing and resting cells permanently and with comparable efficiency, lack of immunogenicity, stable long-term transgene expression, and the ability to transduce the same cells multiple times with rSV40 vectors carrying different transgenes. We have used rSV40 vectors to transduce human cell lines and primary peripheral blood cells, as well as normal human and simian CD34+CD38- bone marrow progenitor cells. Transgenes that have been delivered in this setting include single chain antibodies directed against HIV-1 integrase, HIV-1 reverse transcriptase, and the HIV-1 coreceptor, CXCR4; polyTAR decoys; and RevM10, a dominant negative mutant form of HIV-1 Rev protein. In all cases, transduction efficiencies exceeded 50% without selection, and usually exceeded 95%. Transgene expression was stable in culture and following reimplantation in vivo for the duration of these studies (up to 3 months). Delivered by SV40, all of these transgenes protected unselected susceptible cells from challenge with HIV-1 and, where applicable, SIV. Simian CD34+ cells were transduced with rSV40 vectors carrying polyTAR decoys without selection and induced to differentiate into CD4+ T lymphocytes. These CD4+ derivatives of transduced CD34+ progenitors were highly resistant to challenge with a pathogenic strain of SIV (SIVmac239). Human fetal thymic tissue implanted into SCID mice was transduced in vivo with rSV40 carrying a single chain antibody vs. HIV-1 integrase, then challenged in vivo with HIV. Substantial protection from the HIV-1 challenge was observed. We have, additionally, found that human cell lines can be transduced in sequence and without selection, with rSV40 vectors carrying different anti-HIV-1 transgenes, resulting in >95% of cells stably expressing both transgenes simultaneously. Taken together, these studies suggest that rSV40 vectors may be important and effective delivery vehicles for the transduction of bone marrow progenitor cells and their progeny', particularly with the goal to inhibit infection with immunosuppressive lentiviruses.

379. In Vivo Maturation of U1/Anti-HIV-1 Antisense RNA-expressing CD4+ Cells from Transduced CD34+ PBSC in HIV-1 Seropositive Individuals

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A murine retrovirus-based transducing vector HGTV-43 was developed to deliver three U1/anti-HIV-1 antisense RNA genes into CD34+ cells. This vector is made up of modified MMLV LTR's and three U1 genes in their entirety, each containing a genetic antisense sequence nested within the RNA-coding portion of the gene. The three genetic antisense sequences are complementary to three separate sequences of the HIV-1 genome including one against the TAR region and two against two separate sites of the TAT region. When U937 cells, a line of human promonocyte origin, were transfected to express these three antisense genes and subsequently challenged with HIV-1 (using both patient isolates and laboratory strains) virus production was completely blocked as measured by p24 production and RT-PCR of the input virus. In addition no integration of the HIV-1 viral DNA was detected.

A packaging cell line capable of pseudotyping HGTV-43 with the human T cell leukemia virus II envelope protein was then developed. A stable producer cell line was made by transfecting the clone of the pro-HGTV-43 vector DNA into this packaging cell line. Transducing vector produced from this cell line has consistent titers of greater than 10^7 per ml.

A transduction rate of 25-55% of CD34+ cells has been demonstrated by colony assay in which HGTV-43-transduced CD34+ cells were grown as individual clones to be assayed for antisense RNA expression. Of particular note, the maximum extent of HGTV-43 transduction of CD34+ cells was obtained within the 18-hour period immediately after removal of CD34+ cells from the HIV-1 infected individual and in the absence of factors used to promote cell division including both stromal cell "feeder" layers, and exogenous cytokines such as IL3, IL6 and SCF.

In a clinical trial using this vector, approximately 10^{10} PBMC were collected by leukapheresis from HIV-1 seropositive individuals post granulocyte stimulating factor (GCSF) treatment. After CD34+ cell enrichment on an Isolex 300i column, ~10^8 PBSC (peripheral blood stem cells) were transduced with HGTV-43 followed by autologous infusion without ablation.

Differentiation of transduced CD34+ PBSC within HIV-1 seropositive individuals into PBMC was demonstrated by detection of U1/anti-HIV-1 antisense RNA expression in at least one out of every 10^5 PBMC five months post-infusion. Real-time RT-PCR was performed with cellular RNA isolated from PBMC collected pre-infusion, first week post-infusion, and at monthly intervals thereafter. The identity of U1/anti-HIV-1 antisense RNA-specific RT-PCR amplicon was confirmed by restriction enzyme digestion followed by gel electrophoresis.

More importantly, at five months post-infusion, CD4+ cell maturation from infused CD34+ cells was verified by detection of U1/anti-HIV-1 antisense RNA expression in CD4+-enriched cell populations using real-time RT-PCR. Monitoring patients for the presence of this antisense RNA (in both PBMC and CD4+-enriched PBMC) is ongoing. The efficacy of triple U1/anti-HIV-1 antisense RNA on the inhibition of HIV-1 replication in HIV-1 seropositive individuals is currently under evaluation. These results are a significant and perhaps unique example of the survival of transduced cells within a nonablated adult human subject with continued transgene activity and with replication and differentiation of the transduced CD34+ cell.

380. Applications of MolecularBreeding^tm Technology to Viruses

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There is an acute need for effective technologies to optimize viruses for many clinical applications. Viral vaccines need to be optimized for various properties relating to efficacy, safety and manufacturing issues. Deficiencies in all viral vectors for gene therapy have severely limited their clinical efficacy. Technology for viral optimization is also needed to adapt viruses to grow in model laboratory animals and in tissue culture for use in antiviral drug and vaccine testing (e.g. HIV, Hepatitis B and C). The improved viruses must meet a combination of requirements that the native viruses generally do not possess and this combination of requirements is specific for each application. The absence of an effective technology for optimizing multiple viral properties simultaneously for complex requirements has been a major limitation for engineering viruses. Rational design has occasionally allowed the optimization of single viral properties, but it typically introduces directed mutations and does not broadly search sequence space for sequences that offer the best balance for all the complex requirements. Furthermore, the molecular changes required to obtain desired properties are effectively impossible to predict due to the complex interactions with many other viral and host components. A traditional approach for engineering viruses to obtain specific properties has been to accumulate random point mutations. Viral populations are replicated under selective conditions that favor viruses with point mutations that impart the desired properties. However, phenotypic changes re-